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FINAL REPORT

19990130

INVESTIGATION OF IMMUNOGLOBULIN - INDEPENDENT ANTIMICROBIAL SUBSTANCES IN PORCINE MILK AND COLOSTRUM

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FINAL REPORT FOR THE RESEARCH PROJECT # 19990130

PROJECT TITLE:

Investigation of immunoglobulin-independent antimicrobial substances in porcine milk and colostrum (#19990130)

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SADF FINAL REPORT

PROJECT INFORMATION AND SUMMARY

Title of the project

Investigation of immunoglobulin-independent antimicrobial substances in porcine milk and colostrum (SADF Project #19990130)

Abstract

The intensive farrowing management system with shortened nursing periods results in decreased exposure of piglets to defense components present in maternal milk. A possible result is decreased resistance to infectious diseases manifested during postweaning period. This research program investigated the as yet poorly understood immunoglobulin-independent protective defense mechanisms in porcine colostrum and milk. In particular, it focused on bactericidal, bacteriostatic and bacterial-binding components in porcine colostrum and milk.

We discovered many novel immunoglobulin-unrelated bacterial-binding proteins in porcine milk. Some of these proteins retain their bacterial binding activity even after *in vitro* digestion of milk by gastric an intestinal enzymes. This is potentially a very significant discovery since for many years it has been assumed that immunoglobulins are the only bacterial-binding substances in milk and colostrum.

In addition to that, some proteins digested by enzymes generate cleavage fragments that have potent bactericidal effects similar to antibiotics. These antimicrobial peptides generated by digestion of porcine milk might be a very important protective innate defense of neonatal piglets against pathogenic bacteria. Their potent bactericidal effects are comparable to modern antibiotics. The further investigation of these bactericidal peptides and their feasibility as antibiotic alternatives or enhancers is warranted.

Executive Summary

Conceptual research has been conducted without any deviation from the initial proposal. Schedule of individual research activities has been modified slightly to increase efficiency of this investigation. Substantial amount of novel data have been generated and manuscripts describing our findings have been prepared.

Our study indicated that porcine lactoferrin had distinct immunological differences from bovine lactoferrin. Despite these differences, we hypothesized that digested porcine lactoferrin had antimicrobial activities similar to those of digested bovine lactoferrin. Our experiments demonstrated that pepsin-digested as well as acid-hydrolyzed porcine lactoferrin had similar bactericidal activity as bovine lactoferrin digests. Using various chromatography techniques we isolated bactericidal peptides generated by pepsin digestion of porcine lactoferrin. These peptides have similar molecular weights as previously reported bovine lactoferricin.

Acid-hydrolysis of casein isolated from porcine milk generated antimicrobial activity. Using various chromatographic techniques we isolated bactericidal peptides that have similar molecular weight to bovine casocidin.

These antimicrobial peptides generated by cleavage of proteins of porcine milk (similar to normal gastric digestion in stomach of piglets) might be a very important protective innate

defense of neonatal piglets against pathogenic bacteria. Their potent bactericidal effects are comparable to modern antibiotics. The further investigation of these bactericidal peptides and their feasibility as antibiotic alternatives or enhancers is warranted.

As hypothesized, immunoglobulin-unrelated bacterial-binding proteins have been discovered in porcine colostrum and milk by a batch chromatography using formalin killed F4+ *E. coli*. This is potentially a very significant finding since for many years it has been assumed that immunoglobulins represent the majority of bacterial-binding substances in milk and colostrum. The batch chromatography method employed in these experiments had some undesirable effects which were eliminated by an affinity chromatography method using the AKTA Purifier HPLC system. Initially, comparative investigation of lipopolysaccharide (LPS) binding proteins in porcine milk has been performed by two different affinity chromatography matrices, namely, LPS-Sepharose and LPS-Toyopearl. Subsequently, only LPS-Sepharose matrix was used due to high cost of Toyopearl matrix. Several non-immunoglobulin LPS-binding proteins were discovered and characterized by two-dimensional electrophoresis. LPS binding proteins have been identified by liquid chromatography mass spectrometry. In addition to immunogolobulins, lactoferrin, beta casein, alpha s_2-casein, kappa-casein, alpha s_1-casein and lipoprotein lipogenase were demonstrated to be able to bind LPS of *E. coli* F4. Lactoferrin and beta casein had stronger binding affinity to LPS than the remaining proteins.

We also initiated and completed a new, originally not proposed, but very important investigation focused on immunoglobulin-unrelated proteins binding to fimbriae of F4+ *E. coli*. Bacterial fimbriae are "the arms" attaching bacteria to the intestinal surface. Therefore if "these arms are tied" by fimbrial-binding substances in the milk, infection cannot occur. We isolated F4+ *E. coli* fimbriae. Purified fimbriae were coupled to affinity Sepharose matrix and F4 fimbrial-binding proteins were isolated from porcine milk by affinity chromatography. Several non-immunoglobulin fimbrial F4-binding proteins were discovered and characterized by two-dimensional electrophoresis. Fimbrial F4-binding proteins have been identified by liquid chromatography mass spectrometry. In addition to immunogolobulins, lactadherin, ezrin, moesin, xanthin dehydogenase, alpha lactalbumin, and beta casein were demonstrated to be able to bind fimbria F4 of *E. coli*. Lactadherin and beta casein had stronger binding affinity to fimbria F4 that the remaining proteins.

Our findings demonstrated that in addition to immunoglobulins, porcine milk contains many proteins that bind to bacterial surface components. Although defense functions of the isolated proteins are not known yet, their interaction with *E. coli* F4 might interfere with bacterial attachment and pathogenicity.

In vitro experiments have been designed to determine if lactoferrin and lactadherin prevent or decrease attachment of *E. coli* F4 to HEp-2 cells.

The future conventional or recombinant production of lactogenic innate protective factors and their supplement to diet of early-weaned piglets should increase disease resistance during critical weaning and postweaning periods.

Research team

- Elemir Simko, DVM, DVSc, principal investigator, Department of Veterinary Pathology, WCVM, University of Saskatchewan, 52 Campus Drive Saskatoon, SK, S7N 5B4; Phone: (306) 966-7307, Fax: (306) 966-7439, Email: elemir.simko@usask.ca. This research funding provided by SADF supported in part establishment of Dr Simko's research laboratory investigating pathogenesis of infectious diseases in food animals. Particular interest is in pathogenesis of and resistance to bacterial diseases affecting neonatal and post-weaning piglets. In addition, during the tenure of this funding, Dr. Simko obtained a board certification by the American College of Veterinary Pathologists in 2001 as well as was awarded a tenure at the University of Saskatchewan in 2002. Accordingly, his long term professional career will contribute to the enhancement of quality of food animal production, health and welfare. SADF supported research activities in Dr. Simko laboratory, but not his salary.
- Farshid Shahriar, DVM, MVetSc is a full-time graduate student enrolled in PhD program.

  Dr. Shahriar's stipend has been successfully obtain through a competitive Interprovincial Graduate Student Scholarship. SADF supported in part his research focused on investigation of bacterial-binding and bactericidal substances in porcine milk and colostrum. He obtained extensive knowledge in investigative pathology, acquired many protein biochemistry techniques as well as mastered research methodology and disign. He will defend his thesis in the second half of 2004 and will publish 3-4 papers from his research. In addition, he has developed solid diagnostic pathology skills and will be entering North American (hopefully Canadian) work force as a veterinary pathologist with special interest in diseases of food animals. There has been a great shortage of veterinary pathologist in Canada and USA, and the recent BSE crisis aggravated even more this shortage.
- James E. Talbot, BSc was employed as a full-time research technologist (term position) from Aug 2000 to July 2001, when he accepted a permanent position with NRC - Plant Biotechnology Institute. This was a wise long-term career move for Mr. Talbot and we are happy for him. However, at the same time this event had a transiently negative impact on our productivity. SADF supported in part Mr. Tablot's salary.
- In Nov 2001, Yuanmu Fang DVM, MSc, joined our group as a full-time research technician. Dr. Fang has a solid immunology experience acquired in China. SADF supported in part Dr. Fang's salary.

  In July 2004, he is going to start an MSc program in the Department of Veterinmary Pathology, Western College of Veterinary Medicine. His research will be focused on neonatal diarrhea caused by AIDA-1 *E. coli.*

Funding

We acquired additional funding necessary to bring our expanded research project to completion. In addition to $64,225 obtained from the SADF, Sask Pork contributed $56,445 and Alberta Agriculture Institute $107,825. Additional funding ($99,962) was obtained from the Health Services Utilization and Research Commission to investigate antimicrobial substances in porcine intestinal mucosa and for purchase of a chromatography system. As you can see, for each dollar

obtained from the SADF more than 4 dollars were acquired from other sources for this research program investigating neonatal and postweaning diarrhea in the modern swine production.

TECHNICAL REPORT

Rationale

The intensive farrowing management system with shortened nursing periods results in decreased exposure of piglets to immediate and long-term protection mediated by various defense components present in maternal milk. A possible result is decreased resistance to infectious diseases manifested as an increased incidence of postweaning diarrhea, pneumonia, septicemia and other infectious diseases in weaned piglets and young feeders. This research program investigated the as yet poorly understood immunoglobulin-independent protective defense mechanisms (humoral innate immunity) in porcine colostrum and milk. In particular, it focused on bactericidal, bacteriostatic and bacterial-binding components and their defense function in porcine colostrum and milk. The future conventional or recombinant production of lactogenic innate protective factors and their supplement to diet of early-weaned piglets should increase disease resistance during critical weaning and postweaning periods.

1. Bacteriostatic and bactericidal substances in porcine milk

1.1. Establishment and validation of antimicrobial assays and positive controls

The ultrasensitive radial diffusion and gel overlay assays (Steinberg and Lehrer 1997) have been successfully established in our laboratory using the following positive controls: (i) Polymyxin B, a commercial antibiotic, (ii) ovine neutrophilic defensins isolated according to previously published protocol (Selsted et al. 1993) and (iii) lactoferricin B present in bovine lactoferrin (Sigma-Aldrich Canada Ltd) digested with pepsin as reported by Tomita *et al* (1991) (Figure 1).

1.2. Antimicrobial activity of porcine non-digested milk

Whole colostrum and milk, as well as their white blood cell-free and fat-free fractions obtained by differential centrifugation, were tested for antimicrobial activities using ultrasensitive radial diffusion and disk diffusion assays. Unequivocal inhibition of bacterial proliferation was not detected.

1.3. Antimicrobial activity of porcine digested milk

1.3.1. Porcine lactoferricin

Tomita et al (1991) reported that bovine lactoferrin digested by pepsin has broad-spectrum of antibacterial activity which was later attributed to lactoferricin B (Bellamy et al. 1992). Little is known about porcine lactoferricin, a putative bactericidal peptide in the porcine milk and colostrum. The following investigation has been undertaken to explore structure and activity of porcine lactoferricin.

Polyclonal antibodies against commercially available bovine lactoferrin (Sigma-Aldrich Canada Ltd., Canada) were produced by immunization of rabbits. It was anticipated that

interspecies cross reactivity would be preserved and that polyclonal antibodies against bovine lactoferrin would be employed in immuno-affinity chromatography for isolation and purification of porcine lactoferrin. Unfortunately, the polyclonal anti-bovine-lactoferrin antibodies obtained had very low, if any cross reactivity with porcine lactoferrin (Figure 2). Because of these apparent immunogenic differences between bovine and porcine lactoferrin, we could not use obtained antibodies for our isolation and purification procedures. Despite these differences, we hypothesized that digested porcine lactoferrin had antimicrobial activities similar to those of digested bovine lactoferrin.

Since isolation of porcine lactoferrin was a necessary step for obtaining and studying porcine lactoferricin, an alternative affinity chromatography was employed. Porcine lactoferrin was purified by affinity chromatography from porcine fat-free milk subjected to heparin-sepharose column according to modified protocol by Chu *et al* (1993). Peak 2 in Figure 3 contained a ~ 75 kDa protein thought to be porcine lactoferrin (lane 4 in Figure 4). PAGE migration of this protein was identical to migration of bovine lactoferrin isolated by the same procedure (lane 2 in Figure 2) as well as PAGE migration of commercially available bovine lactoferrin (lane 1 in Figure 2 and lane 5 in Figure 4).

A time-consuming labor-intensive procedure used for isolation of porcine and bovine lactoferrin was replaced by a semi-automated multiple-run chromatographic procedure developed in our laboratory. A sufficient amount of porcine lactoferrin has been purified and characterization of antimicrobial activity against clinically relevant isolates of *E. coli* (F4+ and F41+) was performed. Our numerous experiments demonstrated that pepsin-digested as well as acid-hydrolyzed porcine lactoferrin had similar antimicrobial activity as bovine lactoferrin digests (Figure 5). This antimicrobial activity of porcine lactoferrin generated by pepsin digestion (similar to normal gastric digestion of piglets) might be a very important protective innate defense of neonatal piglets against pathogenic bacteria. Semi-purification of bactericidal lactoferrin fragment(s) generated by pepsin digestion was performed by size exclusion chromatography using Superdex Peptide HR 10/30 column (Amersham Pharmacia Biotech, Sweden). Peak 2 (Fig 6) contained peptide(s) with bactericidal activity (Fig 6). Based on one-dimensional PAGE we suspected that this peak contained several peptides (Fig 7). We were not able to separate these peptides by two-dimensional PAGE because their isoelectric point (pI) is higher than 10 (Fig 7). Therefore, reverse phase chromatography was employed to purify these peptides (Fig 8) and determine that their MW is less than 10 kDa which is consistent with bovine lactoferricin.

1.3.2. Porcine casocidin

Porcine milk was centrifuged at 5000 rpm for 30 minutes at 4 °C to separate and remove fat. Skim milk treatment was performed according to previously reported method (Zucht 1995). Briefly, glacial acetic acid was added to the milk sample at a final concentration of 10% (v/v) and milk was boiled for 5 min. It was cooled to 50 °C and 1 g/l $CaCl_2$ was added. Serine proteases were blocked by 50 μM phenylmethylsufonate. The sample was centrifuged at 5000 rpm for 15 min at 4 °C and supernatant was collected.

The supernatant was diluted 1:4 with distilled water, applied to a strong cation exchange column (Amersham Biosceinces) and eluted by step elution using 25%, 55%, and 95% of 1 M

NaCl pH 3. (Figure 9) Three eluted peaks were collected, dialyzed against distilled water, lyophilized and tested for anti-microbial activity by radial diffusion assay (RDA). The active peak eluted by 95% of 1 M NaCl pH 3 had antimicrobial activity and was subjected to a size exclusion using Superdex 75 chromatography column (Amersham Biosceinces) and 25% acetonitrile and 0.1% TFA elution buffers (Figure 10). RDA revealed one peak with very strong antimicrobial activity (Figure 11). These results indicated that acid hydrolysis of porcine casein generated potent antimicrobial peptides. SDS-PAGE of the fraction with antimicrobial activity demonstrated that there is more than one protein in this fraction. Further purification is being performed to determine which peptides are responsible for antimicrobial activity.

These antimicrobial peptides generated by cleavage of proteins in porcine milk (similar to normal gastric digestion in stomach of piglets) might be a very important protective innate defense of neonatal piglets against pathogenic bacteria. Their potent bactericidal effects are comparable to modern antibiotics. The further investigation of these bactericidal peptides and their feasibility as antibiotic alternatives or enhancers is warranted.

2. Bacterial-binding substances

2.1. Bacterial-binding substances in porcine undigested milk

2.1.1. Bacterial-wall-binding proteins in porcine milk

As hypothesized, several bacterial-binding proteins were discovered in porcine colostrum and milk (Figure 12, lane 2-5) by batch chromatography method (Lu et al. 1996) using formalin killed F4+ *E. coli*. Based on calculated molecular weights, heavy and light immunoglobulin chains were indicated by blue and red arrow heads in skim milk (lane 1 in Figure 12): it was obvious, therefore, that the lactogenic bacterial-binding proteins we eluted from *E. coli* by EDTA, acetylglucosamine, maltose, and mannose (lane 2, 3, 4 and 5, respectively, in Figure 12) were not immunoglobulins. This has a great potential significance since till now most bacterial binding activities have been attributed to immunoglobulins in milk and colostrum.

The batch chromatography method employed in these experiments had two undesirable effects: (i) minor contamination by bacterial proteins and (ii) potential contamination by lactogenic proteins non-specifically bound to bacterial surfaces. Lane 6 (Figure 12), a negative control, contained a few bacterial proteins, indicating that eluted fractions in lanes 2-5 (Figure 12) contained a small amount of bacterial proteins in addition to lactogenic bacterial-binding proteins. Non-specific binding of proteins marked by green boxes in lanes 2-5 (Figure 12) was suspected. Since these proteins represented the most abundant proteins in the milk, non-specific binding could not be ruled out due to, inter alia, limited efficiency of pre-elution washes in the procedures employed. Both of these undesirable effects were eliminated by traditional LPS-affinity chromatography using the AKTA Purifier.

2.1.2. LPS-binding proteins in porcine milk

LPS was isolated from *E. coli* F4+ using Dareau-Hancock procedure (Sprott et al. 1994). Silver-stained, one-dimensional tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) of isolated *E. coli* F4+ LPS revealed a ladder of discrete bands ranging from ~ 37 to 4 kDa (Figure 13) as previously described for most enteric bacteria (Sprott et al. 1994). Similar migration pattern of isolated LPS was also detected by Western blot using

rabbit polyclonal antibodies raised against *E. coli* F4+. The isolated LPS was coupled to two different commercial affinity chromatography matrices, namely, CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech, Sweden) and Toyopearl AF-Epoxy-650M (TosoHaas, PA, USA) according to reported method (Hoover et al. 1998) and LPS-affinity columns were packed. Comparative investigation of LPS-binding proteins in porcine milk have been performed using these two different affinity chromatography matrices. Lactogenic proteins bound to LPS were eluted by either 4M urea or 4M guanidine hydrochloride buffers (Fig 14 and 15). Eluted protein fractions were concentrated and detected by SDS PAGE (Fig 16, lane 1 and 6). Affinity matrices without coupled LPS were used as negative control to identify lactogenic proteins interacting with affinity matrices alone (Fig 13, lane 2 and 5). LPS-binding proteins eluted from LPS-Toyopearl and LPS-Sepharose as well as from both negative columns were characterized by two-dimensional electrophoresis (Figure 17 and 18). Due to high cost of Toyopearl affinity matrix, this study was continued only with Sepharose affinity matrix and LPS-binding as well as Sepharose binding proteins were amino acid sequenced (Table 1).

In addition to the above experiments, LPS-binding proteins eluted from LPS sepharose matrix by 1M NaCl followed by more stringent elution using a low pH buffer of 2.5 were identified by liquid chromatography mass spectrometry. Alpha s_2-casein, κ-casein, alpha s_1-casein and lipoprotein lipogenase were eluted by 1M NaCl from *E. coli* F4 LPS column but not from a control column. Strong binding affinity of lactoferrin and beta casein to LPS was implied by presence of these proteins in low pH elution from the positive columns even after extensive wash and elution by 1M NaCl (Table 2)

2.1.3. Fimbiral-binding proteins in porcine milk

We also initiated and completed a new, originally not-proposed, but very important, investigation focused on immunoglobulin-unrelated proteins binding to fimbriae of F4+ *E. coli*. Bacterial fimbriae are "the arms" attaching bacteria to the intestinal surface. Therefore, if "these arms are tied" by fimbrial-binding substances present in the milk, intestinal colonization and infection cannot occur.

We isolated F4+ *E. coli* fimbriae using a combination of previously published methods (Fang et al. 2000; Korhonen et al. 1980). Coomassie-stained SDS PAGE of isolated F4+ fimbriae revealed a distinct band of ~27 kDa (Figure 16, lane 1-3), consistent with previously reported PAGE migration of F4+ *E. coli* fimbriae (Sun et al. 2000). Western blot using rabbit polyclonal anti-F4+ antibodies (obtained from Dr. J. H. Fairbrother, University of Montreal, Saint Hyacinthe, Quebec) revealed bands with the same PAGE migration pattern (Figure 19, lane 4-6), confirming immunological identity of our isolated fimbriae from F4+ *E. coli*. Purification of F4 fimbriae were performed by size exlusion chromatography using rabbit. Purified fimbriae were coupled to affinity Sepharose matrix and F4 fimbrial-binding proteins were eluted by 1M NaCl followed by more stringent elution using a low pH buffer of 2.5. The eluted fimbrial binding proteins were identified by liquid chromatography mass spectrometry. Lactadherin, ezrin, moesin, xanthin dehydogenase, alpha lactalbumin, and beta casein were eluted by 1M NaCl from *E. coli* F4 fimbrial column but not form a control column. Strong binding affinity of lactadherin and beta casein to F4 fimbria was implied by presence of these proteins in low pH elution from the positive columns even after extensive wash and elution by 1M NaCl (Table 3).

2.2. Bacterial-binding substances in porcine digested milk

Pepsin digestion: Porcine milk was digested according to the previously described method with slight modifications. Briefly, skim milk was adjusted to pH 3.0 with 0.1 N HCl and digested with porcine pepsin (Sigma, St Louis, MO, USA) (20mg/g of milk protein) for 90 min and 37° C in a shaking incubator at 80 rpm. During the digestion, pH was adjusted every 15 min by addition of 0.1 N HCl. At the end of digestion, the pH of hydrolysis mixture was raised to pH 7.5 with 2M NaOH to inactivate the pepsin enzyme. The mixture was centrifuged at 3600 xg for 15 min and supernatant was collected for affinity chromatography.

Pancreatin digestion: Following hydrolysis with pepsin, pancreatin enzyme (Sigma, St Louis, MO, USA) was added at 40mg/g of milk protein and incubated for 60 min at 37° C in a shaking incubator at 80 rpm. Pancreatin enzymes were inactivated by heating for 5 min at 95° C in a water bath. The sample was cooled to room temperature, centrifuged at 3600 xg for 15 min and supernatant was collected for affinity chromatography.

Affinity chromatography using LPS sepharose matrix was performed using the same protocol as for non-digested milk. The following two tables summarize the results of these experiments.

LPS *E. coli* F4 biding proteins isolated from pepsin digested porcine milk. Proteins were identified by LC-MS.

	LPS column		**Control column**	
	NaCl elution	*pH 2.5 elution*	*NaCl elution*	*pH 2.5 elution*
Porcine LF	+	+	-	-
Kappa Casein	+	-	-	-
Alpha s_1 Casein	-	+	-	-

LPS *E. coli* F4 biding proteins isolated from pepsin plus pancreatin digested porcine milk. Proteins were identified by LC-MS.

	LPS column		**Control column**	
	NaCl elution	*pH 2.5 elution*	*NaCl elution*	*pH 2.5 elution*
Alpha Lactalbumin	+	-	-	-
Structure of porcine Beta Lactoglobulin	+	+	-	-

The above-indicated findings demonstrate that in addition to immunoglobulins, digested and non-digested porcine milk contains many proteins that bind to bacterial surface components such as fimbria and LPS both of which are important virulent and pathogenic factors of bacteria. Although defense functions of the isolated proteins are not known yet, their interaction with *E. coli* F4 might interfere with bacterial attachment and pathogenicity. *In vitro* experiments have been designed to determine if lactoferrin and lactadherin prevent or decrease attachment of *E. coli* F4 to HEp-2 cells.

ACKNOWLEDGEMENT

Saskatchewan Department of Agriculture and Food was acknowledged after each presentation and on each poster presentation (see attached copies). In addition to that, the Saskatchewan Department of Agriculture and Food will be acknowledged in each manuscript published in peer-reviewed journals and reprints of these manuscript will be submitted to the Saskatchewan Department of Agriculture and Food once they are available.

PROJECT DEVELOPED MATERIALS (DISSEMINATION / COMMUNICATION OF RESULTS)

Seminars and Presentations

1. F. Shahriar and E. Simko: Innate anti-bacterial defenses in porcine milk; 2003 Canadian Association of Veterinary Pathologist Annual Meeting, Winnipeg, Manitoba, July 2003
2. F. Shahriar: Innate anti-bacterial defenses in porcine milk, Ph.D. Research Progress Seminar; Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada, March 19, 2003
3. F. Shahriar: Innate anti-bacterial defenses in porcine milk, Ph.D. Research Progress Seminar; Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada, March 6, 2002
4. F. Shahriar: Immunoglobulin independent anti-microbial substances in porcine colostrum/milk, Ph.D. Research Proposal Seminar; Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada, March 16, 2001

Posters and/or Abstracts

1. F. Shahriar, M. Ngeleka, J.R. Gordon, E. Simko, (2003). Non-immunoglobulin *Escherichia coli* F4-binding proteins in porcine milk. 54th Annual Meeting of the American College of Veterinary Pathologists, Banff, Canada – Poster and Abstract (Copy attached)
2. F. Shahriar, M. Ngeleka, J.R. Gordon, E. Simko: *Escherichia coli* fimbrial-binding proteins in porcine milk; WCVM Graduate Student Poster Day, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada, March 14, 2003 (Copy attached)
3. F. Shahriar, S. Shah, J. E. Talbot, E. Simko: *Escherichia coli* LPS-binding proteins in porcine milk: CIHR National Health Research Poster Competition, Faculty of Medicine, University of Manitoba, June 5, 2002. (Copy attached)
4. F. Shahriar, E. Talbot, E. Simko: Isolation of an anti-bacterial peptide from pepsin-digested porcine lactoferrin; WCVM Graduate Student Poster Day, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada, January 25, 2002 (Copy attached)
5. F. Shahriar, S. Shah, J. E. Talbot, E. Simko: *Escherichia coli* LPS-binding proteins in porcine milk; Graduate Student Poster Day, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada, March 16, 2001 (Copy attached)

REFERENCES

Bellamy, W., Takase, M., Wakabayashi, H., Kawase, K. and Tomita, M., Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin, J Appl Bacteriol, 73 (1992) 472-9.

Chu, R.M., Wang, S.R., Weng, C.N. and Pursel, V.G., Isolation and characterization of porcine milk lactoferrin, American Journal of Veterinary Research, 54 (1993) 1154-1159.

Fang, L., Gan, Z., Marquardt, R.R., Fang, L. and Gan, Z.B., Isolation, affinity purification, and identification of piglet small intestine mucosa receptor for enterotoxigenic Escherichia coli K88ac+ fimbriae, Infection and Immunity, 68 (2000) 564-569.

Hoover, G.J., el Mowafi, A., Simko, E., Kocal, T.E., Ferguson, H.W. and Hayes, M.A., Plasma proteins of rainbow trout (*Oncorhynchus mykiss*) isolated by binding to lipopolysaccharide from *Aeromonas salmonicida*, Comp Biochem Physiol B, 120 (1998) 559-69.

Korhonen, T.K., Nurmiaho, E.L., Ranta, H. and Eden, C.S., New Method for isolation of immunologically pure pili from Escherichia coli, Infect Immun, 27 (1980) 569-75.

Lu, J., Tay, P.N., Kon, O.L. and Reid, K.B., Human ficolin: cDNA cloning, demonstration of peripheral blood leucocytes as the major site of synthesis and assignment of the gene to chromosome 9, Biochem J, 313 (1996) 473-8.

Saito, H., Miyakawa, H., Tamura, Y., Shimamura, S. and Tomita, M., Potent bactericidal activity of bovine lactoferrin hydrolysate produced by heat treatment at acidic pH, Journal of Dairy Science, 74 (1991) 3724-3730.

Selsted, M.E., HPLC methods for purification of antimicrobial peptides, Methods Mol Biol, 78 (1997) 17-33.

Selsted, M.E., Tang, Y.Q., Morris, W.L., McGuire, P.A., Novotny, M.J., Smith, W., Henschen, A.H. and Cullor, J.S., Purification, primary structures, and antibacterial activities of beta-defensins, a new family of antimicrobial peptides from bovine neutrophils, J Biol Chem, 268 (1993) 6641-8.

Sprott, G.D., Koval, S.F. and Schnaitman, C.A., Cell fractionation. In: P. Gerhardt, R.G.E. Murray, W.A. Wood and N.R. Krieg (Eds.), Methods of general and molecular bacteriology, American Society for Microbiology, Washington, D.C., 1994, pp. 72-103.

Steinberg, D.A. and Lehrer, R.I., Designer assays for antimicrobial peptides. Disputing the "one-size-fits-all" theory, Methods Mol Biol, 78 (1997) 169-86.

Sun, R., Anderson, T.J., Erickson, A.K., Nelson, E.A. and Francis, D.H., Inhibition of adhesion of Escherichia coli K88ac fimbria to its receptor, intestinal mucin-type glycoproteins, by a monoclonal antibody directed against a variable domain of the fimbria, Infection and Immunity, 68 (2000) 3509-3515.

Tomita, M., Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H. and Kawase, K., Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin, Journal of Dairy Science, 74 (1991) 4137-4142.

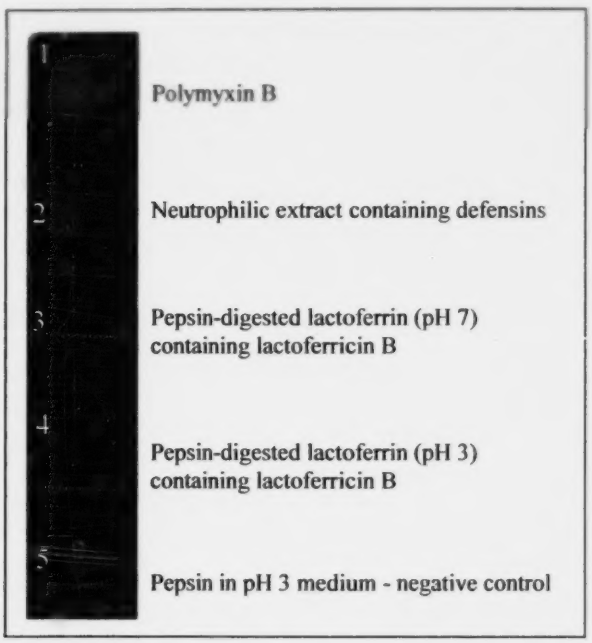

Figure 1 – Coomassie-stained radial diffusion assay of antimicrobial activity of Polymyxin B (well 1), neutrophilic defensins (well 2), pepsin-digested bovine lactoferrin in pH 7 (well 3) and pH 3 (well 4). The well 5 is negative control.

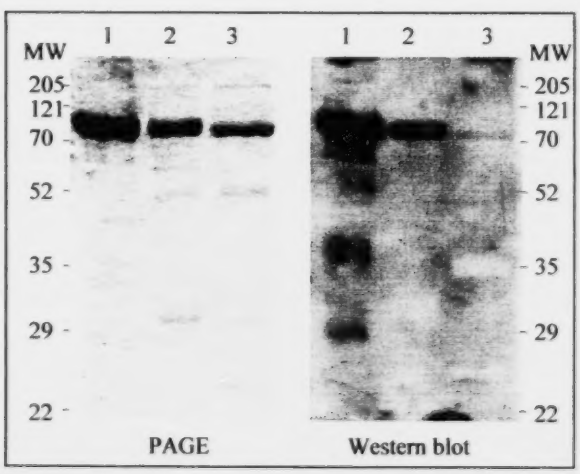

Figure 2 - PAGE of commercial bovine lactoferrin (lane 1) as well as bovine (lane 2) and porcine (lane 3) lactoferrin isolated by heparin affinity chromatography. Western blot of the identical PAGE using rabbit anti-bovine-lactoferrin polyclonal antibodies demonstrates lack of detection of porcine lactoferrin.

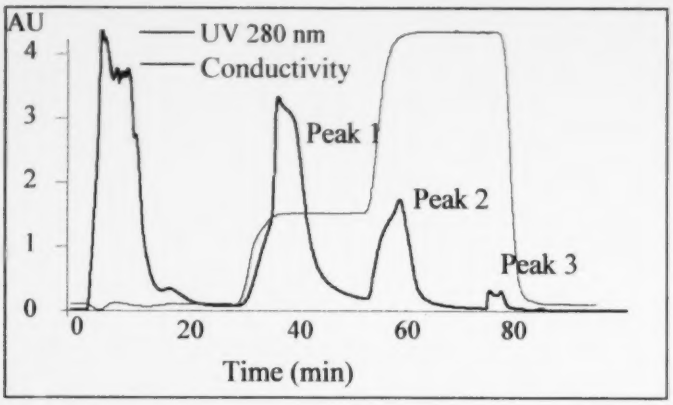

Figure 3. Elution patterns of porcine lactoferrin from a heparin-sepharose column. Peak 2 contains crude lactoferrin

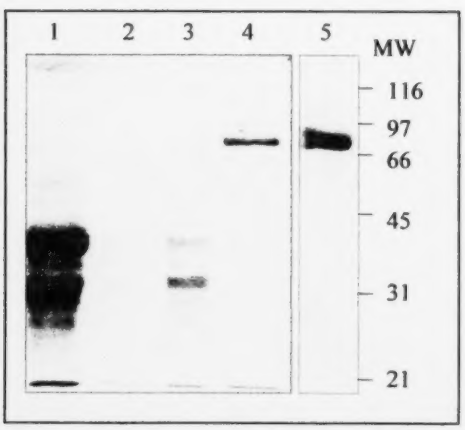

Figure 4. PAGE of proteins eluted from heparin column (Fig. 3): Proteins eluted in Peak 1 and 3 are in lane 1-3. Peak 2 contains porcine lactoferrin in lane 4. Commercial bovine lactoferrin is in lane 5.

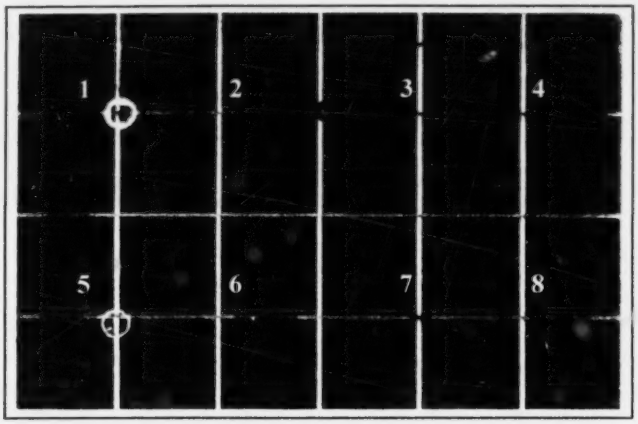

Figure 5. Non-stained radial diffusion gel demonstrating comparative antibacterial activity of bovine and porcine lactoferrins against clinically relevant *E. coli* F4+: Acid hydrolysis digests of both bovine (well 1) and porcine (well 4) lactoferrins as well as pepsin-digested bovine (well 2) and porcine (well 6) lactoferrins have similar antibacterial activities as evident by bacterial growth inhibition zone (red arrowhead) around the wells (blue arrowhead) (Pepsin was inactivated by heating to 80°C for 15 min after 4 hr digestion). When heat inactivation of pepsin activity was not performed neither bovine (well 3) nor porcine (well 7) lactoferrin digest had antimicrobial activity.

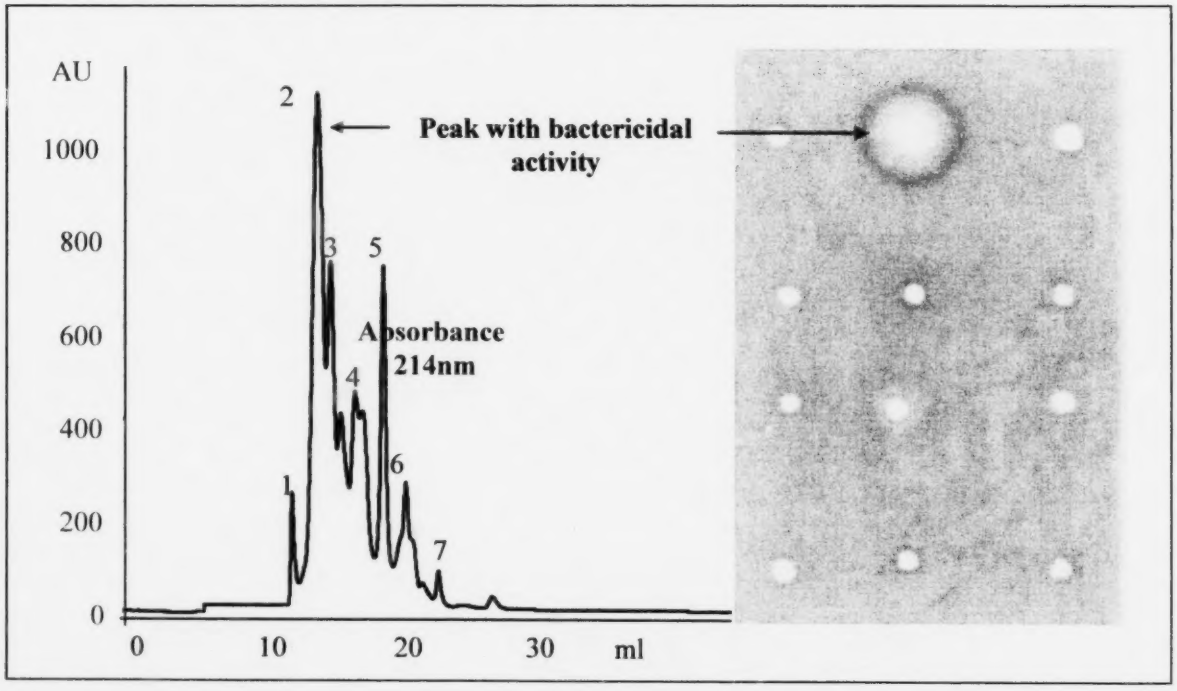

Figure 6. Separation of pepsin-digested fragments of porcine lactoferrin based on their molecular weight using Superdex Peptide HR 10/30 column. Peak 2 indicated by green arrow has bactericidal activity confirmed by radial diffusion on the right side.

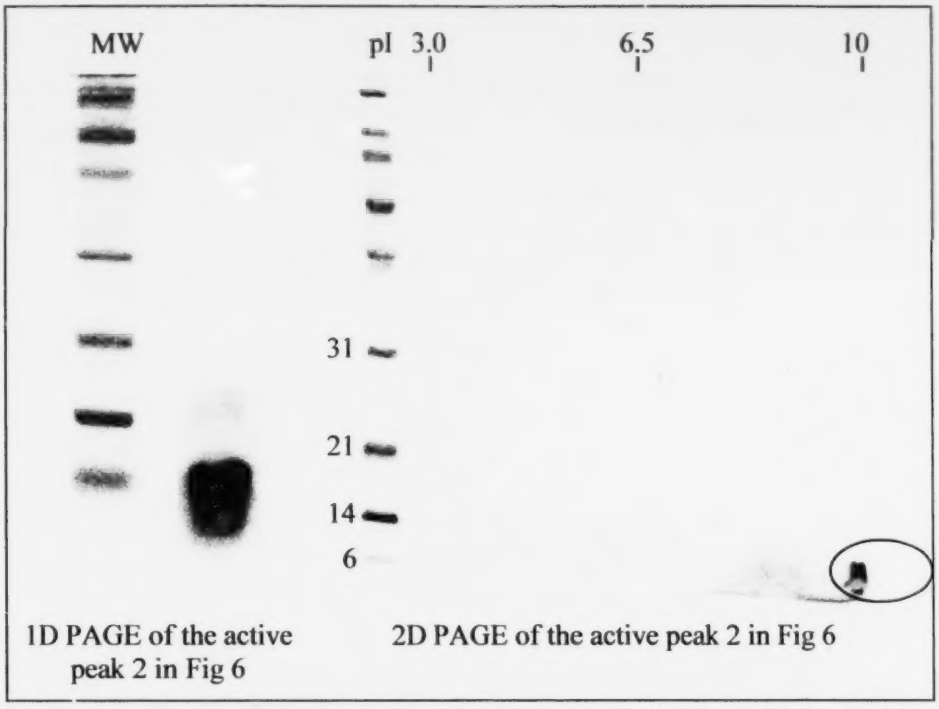

Figure 7. One and two dimensional PAGE separation of bactericidal peptides isolated in peak 2 in Figure 6.

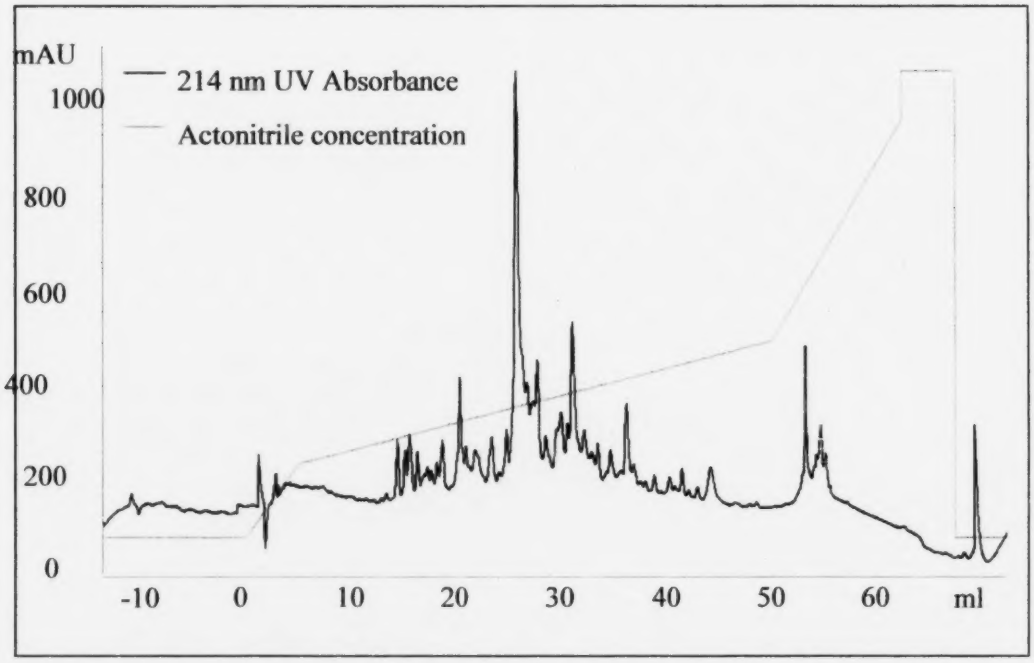

Figure 8. Reverse phase chromatographic separation of bactericidal peptides isolated in Peak 2 in Figure 6.

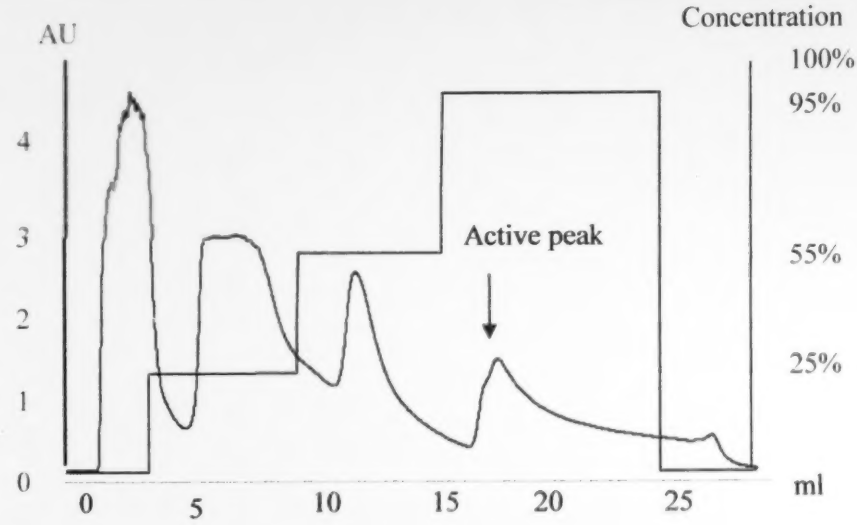

Figure 9: Ion exchange chromatography. Milk sample was applied to a strong cation exchange chromatography column in three steps elution by 1M NaCl pH 3. Eluted peak with 95% concentration (arrow) NaCl demonstrated anti-microbial activity.

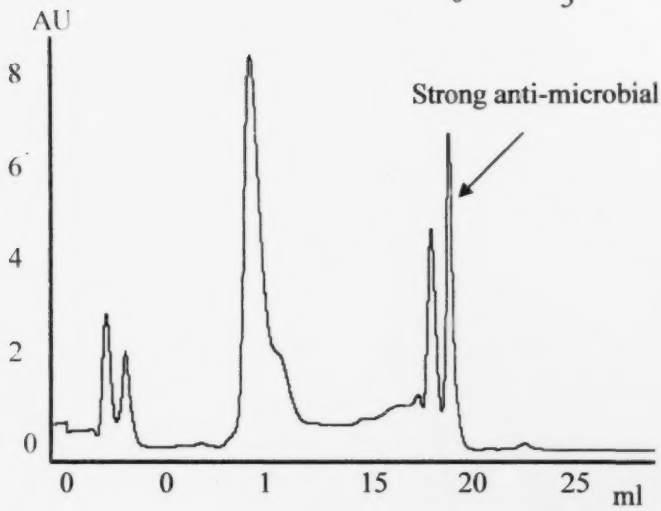

Figure 10: Size exclusion chromatography. Proteins in active peak from cation exchange column were fractionated by a superdex 75 size exclusion column and tested for anti-microbial activity. The peak indicated by arrow showed strong anti-microbial activity.

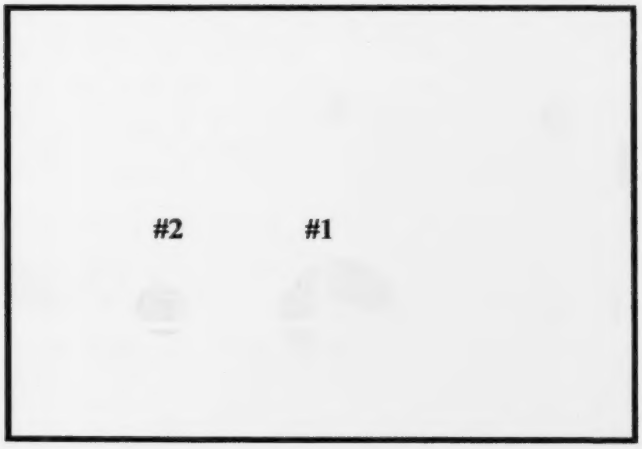

Figure 11: RDA of eluted peaks form size exclusion Superdex 75 column (Fig 10). Strong anti-microbial activity is shown by large clear zone (#1) that corresponds to the peak indicated in the Fig 10. #2 clear zone represents antimicrobial activity by 10 mirol of porcine lactoferrin (50mg/ml)

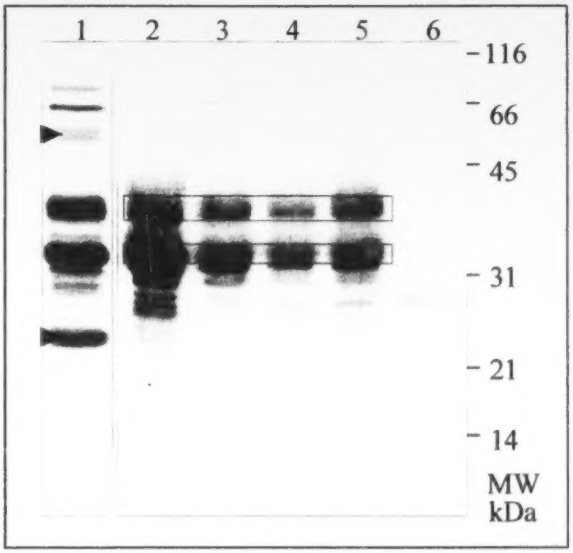

Figure 12. Porcine lactogenic bacterial-binding proteins. Porcine skim milk collected at day 21 of lactation (lane 1); Proteins eluted from F4+ E. coli by EDTA, N-acetyl-D-glucosamine, maltose, and mannose lane 2, 3, 4, and 5, respectively.

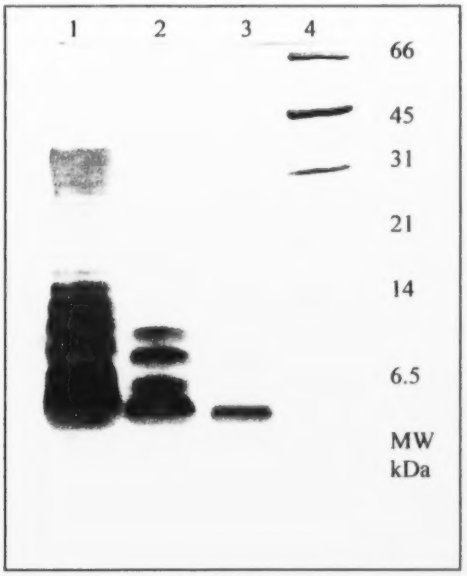

Figure 13. Silver-stained tricine SDS PAGE containing 5, 1 and 0.5 μg of F4+ *E. coli* LPS in lane 1, 2, and 3, respectively. BioRad low range molecular weight markers (lane 4)

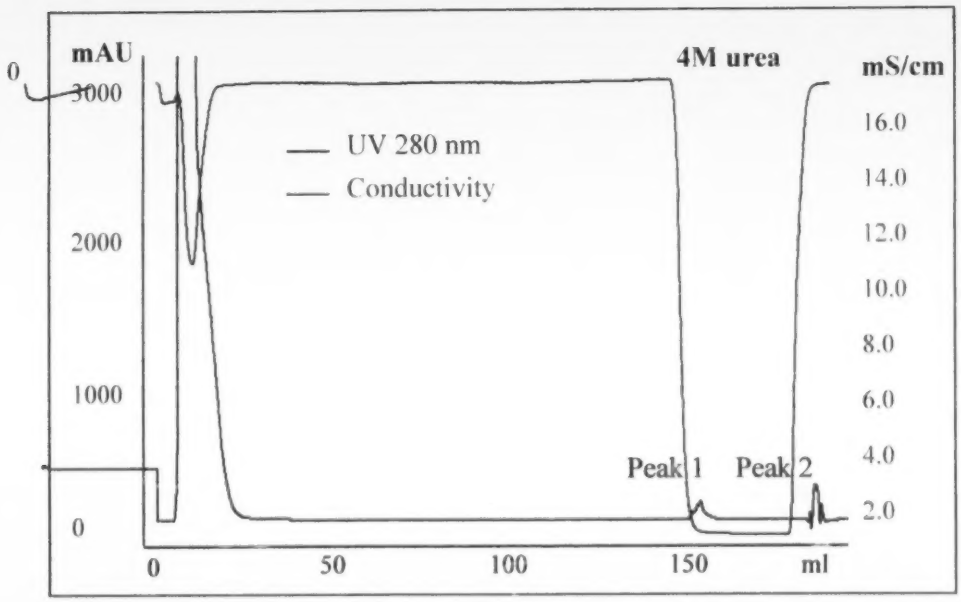

Figure 14. Elution patterns of porcine lactogenic LPS-binding proteins (peak 2) from LPS-sepharose column by 4M urea.

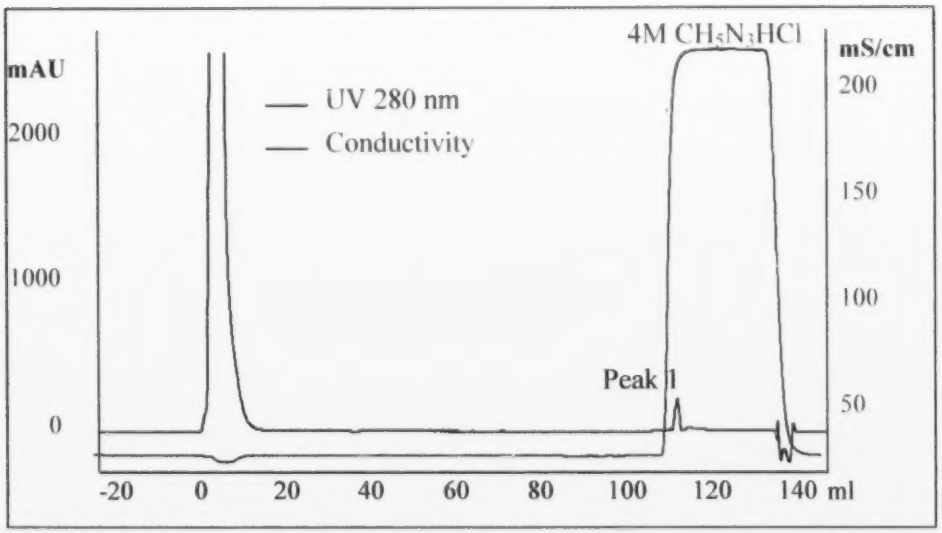

Figure 15. Elution patterns of porcine lactogenic LPS-binding proteins (peak 1) from LPS-Toyopearl column by 4M guanidine hydrochloride.

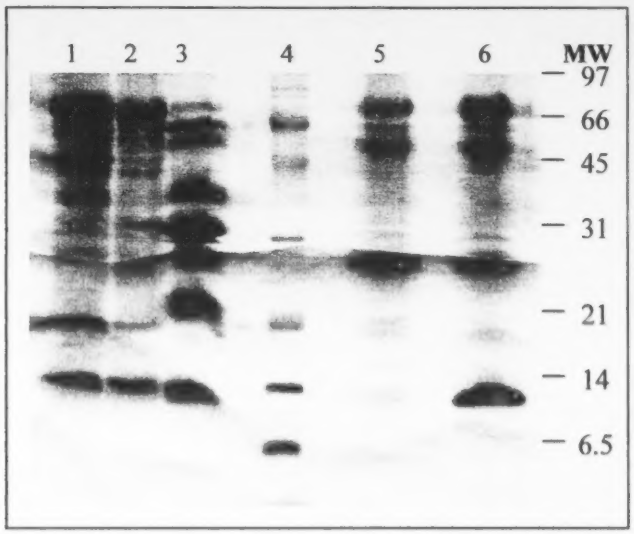

Figure 16 – One-dimentional SDS-PAGE separated proteins eluted from LPS-Toyopearl (lane 1), Toyopearl (lane 2), LPS-Sepharose (lane 4), and Sepharose. Fat-free milk is separated in lane 3 and molecular weight markers in lane 4.

P.S. We apologize for poor quality of this image.

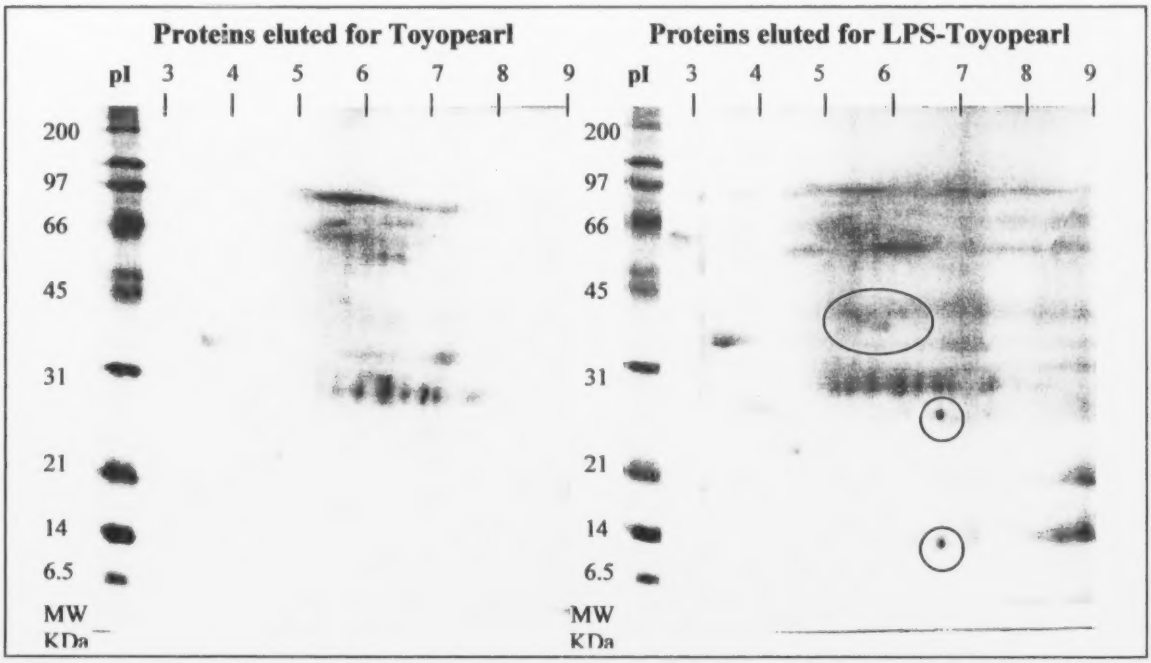

Figure 17 – Two-dimensional SDS PAGE separation of proteins eluted from LPS-Toyopearl and Toyopearl negative column. Proteins in circled areas have apparent LPS specific affinity.

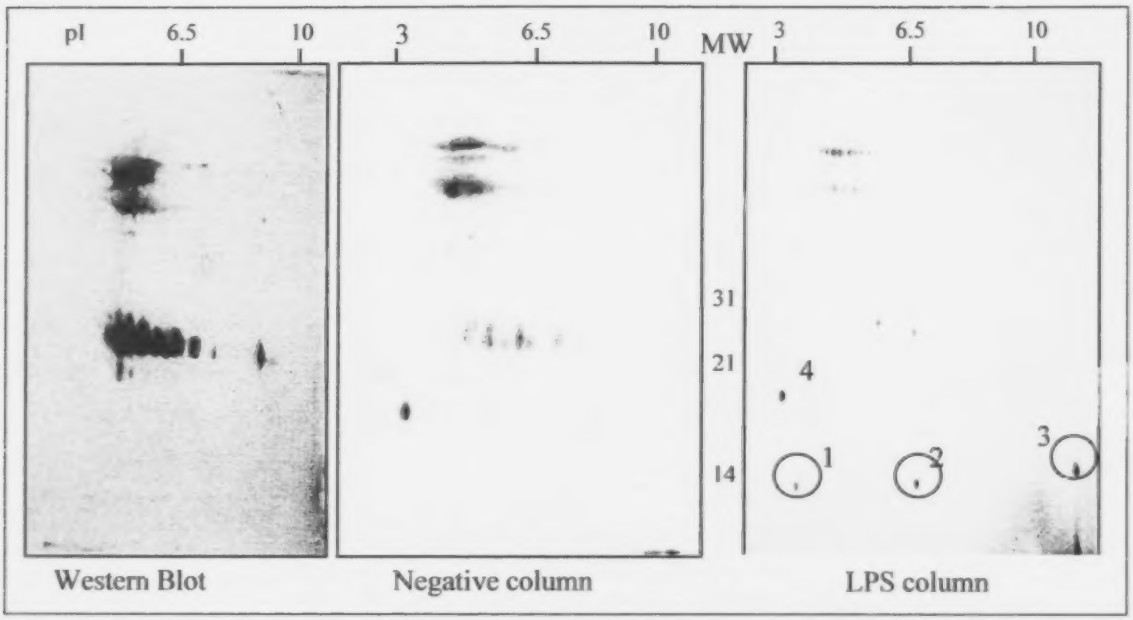

Figure 18. 2D PAGE separation of proteins eluted from LPS-Sepharose (positive) and Sepharose (negative) column. Circled proteins are LPS-binding proteins. These proteins are not immunoglobulins as demonstrated by Western blot using anti-porcine IgG antibodies. Numbered proteins were submitted for amino acid sequencing (See Fig 16)

Table 1. Amino acid sequences and identity of proteins identified by numbers in Figure 15

Protein in Fig 15	AA sequences	Identity
Protein 1	CELSQVLK NLCGLSCDK FLDDDLTDDMMCAK	Alpha-Lactalbumin
Protein 2	LFQENGELV VTDWLK SFLGEASA MDFQENGELLEDQFVK	Not determined
Protein 3	TLDNDLMLLR DNDLTNGDF LGASFLK VTDLFK	Not determined
Protein 4	DVTPLMTELDTQK RLLPAQLEEQC	Beta-lactoglobulin
Protein 4	WQDQPNVYPPTVAR RQFLPYPYYAR KYLPLYYFLNR	Kappa Casein

Table 2. Non-immunoglobulin LPS-binding proteins in porcine milk eluted from LPS-sepharose but not from sepharose matrices by 1M NaCl and low pH buffers. Eluted proteins were identified by Liquid Chromatography Mass Spectrometry

	LPS column		Control column	
	NaCl elution	Low pH elution	NaCl elution	Low pH elution
Lactoferrin	+	+	+	-
β- Casein	+	+	+	-
α-S2 Casein	+	-	-	-
κ- Casein	+	-	-	-
α-S1 Casein	+	-	-	-
Whey acidic prot.	+	-	-	-
Lipoprot. Lipase	+	-	-	-

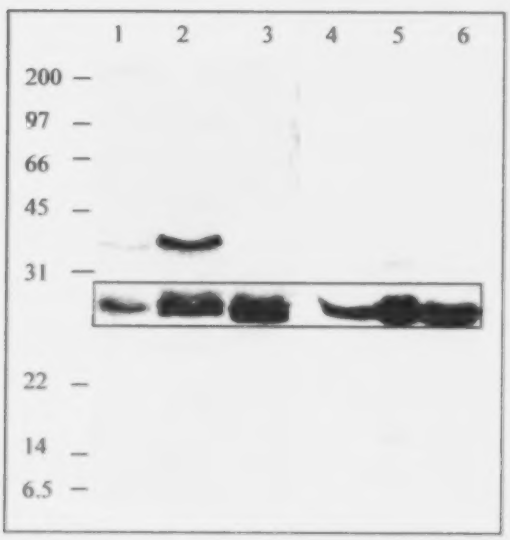

Figure 19 Coomassie-stained SDS PAGE and Western blot of isolated F4+ fimbriae from *E. coli*. Crude fimbrial preparation (lane 1 and 4), ammonium (lane 2 and 5) and citric acid (lane 3 and 6) precipitated/purified F4+ fimbriae.

Table 3. Non-immunoglobulin F4 fimbria-binding proteins in porcine milk eluted from F4-sepharose but not from sepharose matrices by 1M NaCl and low pH buffers. Eluted proteins were identified by Liquid Chromatography Mass Spectrometry

	LPS column		Control column	
	NaCl elution	Low pH elution	NaCl elution	Low pH elution
Lactadherin	+	+	-	-
β- Casein	+	+	-	-
Ezrin	+	-	-	-
Moesin	+	-	-	-
α- lactalbumin	+	-	-	-
Xanthin dehyd	+	-	-	-

Escherichia coli LPS-binding proteins in porcine milk

F. Shahriar, S. Shah, J. E. Talbot, E. Simko

Department of Veterinary Pathology

Western College of Veterinary Medicine

Introduction

The intensive farrowing management system with shortened nursing periods results in decreased exposure of piglets to various defense components present in maternal milk [1,2]. A possible consequence is decreased resistance to diseases, manifested as an increased incidence of postweaning infections. Immunoglobulin-independent innate defenses in porcine milk are not well understood.

We examined porcine milk for non-immunoglobulin proteins that bind to LPS of *E. coli* F4, an important enteric pathogen in piglets.

Materials and Methods

Affinity chromatography: Lipopolysaccharide (LPS) isolated from *E.coli* F4 was coupled to Toyopearl Epoxy affinity matrix (Tosohaas, PA) [3]. Milk proteins bound to LPS were eluted by 0.5 M NaCl (Fig 1). Affinity matrix alone was used as negative control to identify nonspecifically bound milk proteins to the matrix.

Protein detection: Eluted protein fractions were detected by Coomassie blue stain after polyacrylamide gel electrophoresis (PAGE). Western blot was used to distinguish eluted immunoglobulins from the other LPS-binding proteins in porcine milk.

Results

Four LPS-binding proteins with approximate molecular weights of 16, 23, 45, and 48 kDa (Fig 2, lane 3) were found in porcine milk.

These proteins were not related to immunoglobulins in porcine milk as demonstrated by lack of detection by anti-porcine-immunoglobulin antibodies in Western blot (Fig 2, lane 4).

There were several similar proteins eluted from both LPS-Toyopearl and Toyopearl columns. Since some of them are more abundant in elution from LPS column, we suspect that these proteins bind LPS as well as Toyopearl matrix.

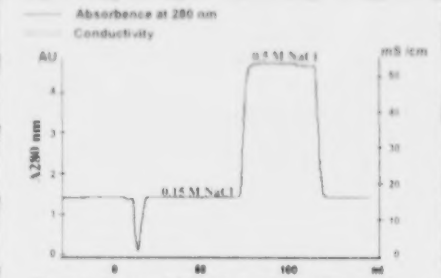

Figure 1 - Toyopearl-LPS column: Elution pattern of LPS-binding proteins in porcine milk

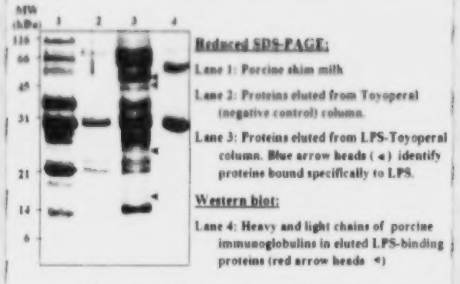

Figure 2 - Detection of LPS-binding proteins in porcine milk

Conclusion

This study demonstrated that, in addition to immunoglobulins, porcine milk has a repertoire of LPS-binding proteins that might be involved in innate defense against infectious diseases in suckling piglets.

Identification, characterization and determination of biological function of these proteins as well as other bacterial surface binding proteins in porcine milk is being performed.

References

1. Fairbrother JM. Enteric colibacillosis. In: Leman AD, et al. eds. Diseases of swine. 7th ed. Ames: Iowa State University Press, 1992: 489-497
2. Xanthou M. Immune protection of human milk. Biology of the Neonate 1998; 74(2):121-133
3. Hoover GJ, et al. Plasma proteins of rainbow trout (*Oncorhychus mykiss*) isolated by binding to lipopolysaccharide from *Aeromonas salmonicida*. Comparative Biochemistry and Physiology (B) 1998;120(3):559-56

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Saskatchewan Agriculture Development Fund

Sask Pork

Alberta Agriculture Research Institute

Interprovincial Graduate Student Fellowship

Isolation of an anti-bacterial peptide from pepsin-digested porcine lactoferrin

F. Shahriar, J. Talbot & E. Simko; Department of Veterinary Pathology; University of Saskatchewan

Introduction

The high incidence of infectious diseases and diarrhea in weaned piglets as well as progressively shortened nursing period in modern industry point towards the importance of milk in disease control and prevention. Lactoferrin (LF), an iron-binding glycoprotein, has a broad spectrum of anti-microbial activities mediated frequently by iron sequestration. In addition, pepsin digestion of bovine LF generates a potent bactericidal peptide that kills bacteria by iron-independent means[1].

The purpose of this study was to determine if porcine LF digested by pepsin generates peptide(s) with anti-microbial activities against a clinical isolate of enterotoxigenic *Escherichia coli* .

Material and Methods

Porcine LF isolation: Porcine LF was purified from porcine skim milk by affinity chromatography using HI-Trap heparin HP columns (Amersham Pharmacia Bio-Sciences)[1,2].

Porcine LF digestion: Lyophilized porcine LF was digested in vitro with porcine pepsin (Sigma Chemical Co.) as previously described[2].

Fractionation of peptides: Digested peptides were fractionated based on molecular weight using a size exclusion Superdex peptide HR 10/30 column (Amersham Pharmacia Bio-Sciences).

Anti-microbial assay: Radial diffusion assay was used to assess the activity of different fractions of digested porcine LF against *Escherichia coli* F4[3].

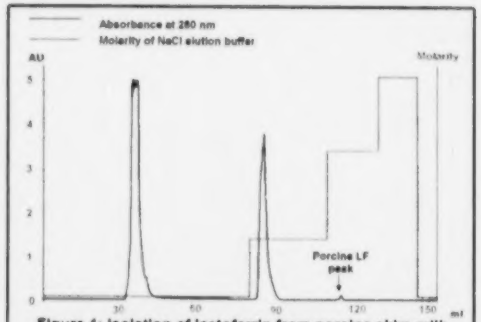

Figure 1: Isolation of lactoferrin from porcine skim milk by affinity chromatography using a heparin column.

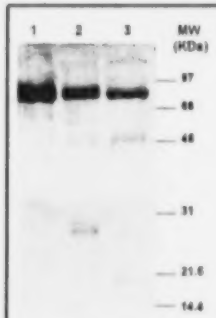

Figure 2: SDS-PAGE of commercial bovine lactoferrin (Lane 1), and of isolated bovine (Lane 2) and porcine (lane 3) lactoferrin.

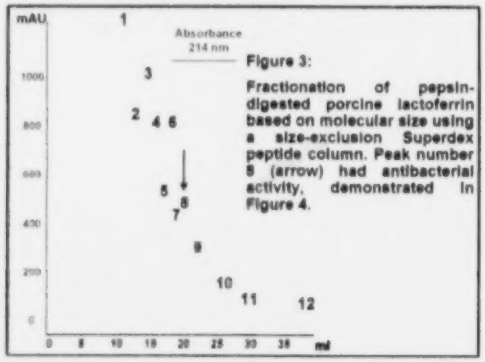

Figure 3: Fractionation of pepsin-digested porcine lactoferrin based on molecular size using a size-exclusion Superdex peptide column. Peak number 8 (arrow) had antibacterial activity, demonstrated in Figure 4.

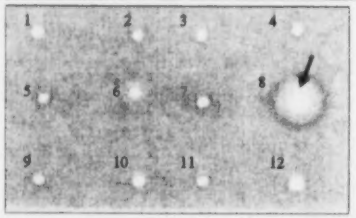

Figure 4. Coomassie-stained radial diffusion gel demonstrating anti-microbial activity of fractionated digested porcine lactoferrin. The digested fraction obtained from peak 8 (Figure 3) contains potent inhibitor(s) of bacterial growth, indicated by a large clear zone (arrow) around well 8.

Results

Porcine lactoferrin was eluted from Heparin-Sepharose matrix by 1 M NaCl (Figure 1) in relatively pure form (Figure 2, Lane 3). Electrophoretic migration of isolated porcine lactoferrin was similar to bovine commercial and isolated lactoferrin (Figure 2).

Pepsin digested porcine lactoferrin was fractionated based on molecular size (Figure 3): one fraction (Peak 8) contained a potent inhibitor(s) of bacterial growth (Figure 4).

Conclusion

Pepsin digestion of porcine lactoferrin generates a potent anti-bacterial peptide(s). It is hypothesized that this peptide together with other anti-microbial substances in milk is involved in defense against infectious agents including intestinal pathogens in suckling piglets.

Characterization and determination of biological function of this peptide(s) are underway.

Funding

Saskatchewan Agriculture Development Fund

Alberta Agriculture Research Institute

Sask Pork

Interprovincial Graduate Student Fellowship

Escherichia coli LPS-binding proteins in porcine milk

Farshid Shahriar, Sunil Shah, James E. Talbot, Elemir Simko

Department of Veterinary Pathology

University of Saskatchewan

Introduction

Recognition and binding to pathogens are crucial steps of the host defense against infectious diseases. These protective effects have been recognized in colostrum and milk and attributed predominantly to colostral and lactogenic immunoglobulins. Since there are many other non-immunoglobulin proteins in milk, objective of this study was to investigate non-immunoglobulin proteins in porcine milk that bind to LPS of *Escherichia coli* F4, an important enteric pathogen in piglets

Materials and Methods

Affinity chromatography: Lipopolysaccharide (LPS) isolated from *E.coli* F4 was coupled to CNBr Activated Sepharose 4B (Amersham Pharmacia Bio-Sciences) and subjected to 21 day fat-free porcine milk. Proteins bound to the LPS were eluted by 4 M Urea (Fig. 1). Affinity matrix alone was used as negative control to identify nonspecifically bound milk proteins to the matrix.

Protein detection: Eluted protein fractions were concentrated and detected by 2-dimensional electrophoresis (Fig. 2). Western blot was used to distinguish eluted immunoglobulins from the other LPS-binding proteins in porcine milk (Fig. 3).

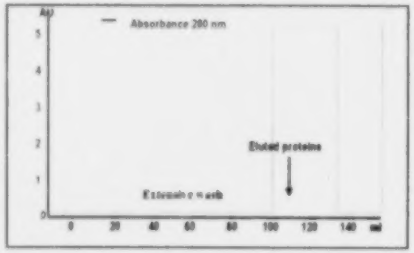

Figure 1: Isolation of LPS-binding proteins from porcine milk by affinity chromatography

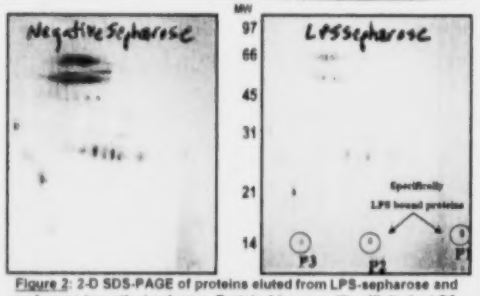

Figure 2: 2-D SDS-PAGE of proteins eluted from LPS-sepharose and sepharose (negative) columns. Protein 1 has specific affinity for LPS, whereas proteins 2 and 3 have affinity for both LPS and Sepharose.

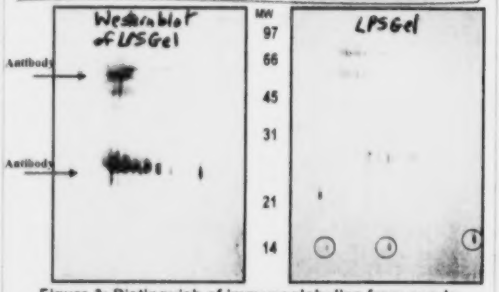

Figure 3: Distinguish of immunoglobulins from non-Ig proteins by western blot of eluted proteins from LPS column

Results

1. There is one non-immunoglobulin protein in porcine milk that specifically bind to the LPS of *E.coli* F4. This protein has molecular weight of about 14 KDa and isoelectric point >11 (proteins number 1 on Fig. 2). Characterization of this protein is underway
2. Two other proteins (protein number 2 and 3 on Fig. 2) have strong attachment to the LPS than sepharose beads. Protein number 3 is lactoglobulin and characterization of protein numbe 2 is being performed.
3. Several other proteins bind to the sepharose beads and possibly to the LPS of *E.coli* F4.

Conclusion

This study demonstrated that, in addition to immunoglobulins, porcine milk has a repertoire of non-immunoglobulin LPS-binding proteins that may involve in innate defense against enteric diseases in suckling piglets.

Fundings

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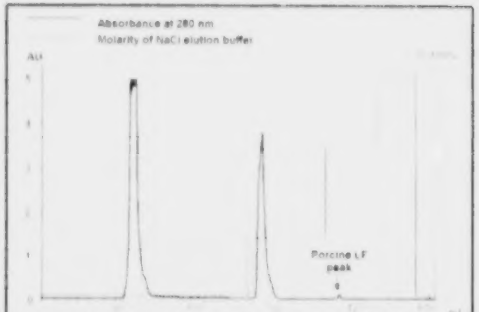

Figure 1: Isolation of lactoferrin from porcine skim milk by affinity chromatography using a heparin column.

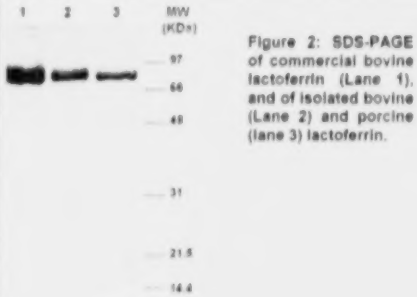

Figure 2: SDS-PAGE of commercial bovine lactoferrin (Lane 1), and of isolated bovine (Lane 2) and porcine (lane 3) lactoferrin.

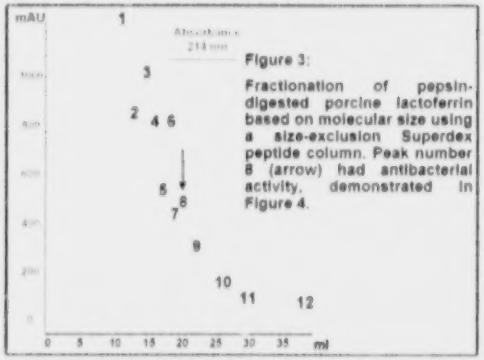

Figure 3:

Fractionation of pepsin-digested porcine lactoferrin based on molecular size using a size-exclusion Superdex peptide column. Peak number 8 (arrow) had antibacterial activity, demonstrated in Figure 4.

Figure 4. Coomassie-stained radial diffusion gel demonstrating anti-microbial activity of fractionated digested porcine lactoferrin. The digested fraction obtained from peak 8 (Figure 3) contains potent inhibitor(s) of bacterial growth, indicated by a large clear zone (arrow) around well 8.

Results

Porcine lactoferrin was eluted from Heparin-Sepharose matrix by 1 M NaCl (Figure 1) in relatively pure form (Figure 2, Lane 3). Electrophoretic migration of isolated porcine lactoferrin was similar to bovine commercial and isolated lactoferrin (Figure 2).

Pepsin digested porcine lactoferrin was fractionated based on molecular size (Figure 3): one fraction (Peak 8) contained a potent inhibitor(s) of bacterial growth (Figure 4).

Conclusion

Pepsin digestion of porcine lactoferrin generates a potent anti-bacterial peptide(s). It is hypothesized that this peptide together with other anti-microbial substances in milk is involved in defense against infectious agents including intestinal pathogens in suckling piglets.

Characterization and determination of biological function of this peptide(s) are underway.

Funding

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Interprovincial Graduate Student Fellowship

Escherichia coli LPS-binding proteins in porcine milk

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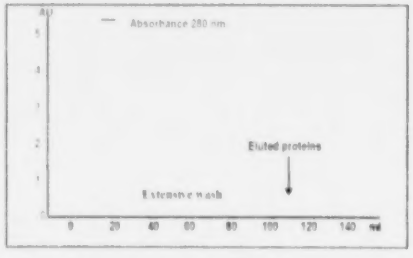

Figure 1: Isolation of LPS-binding proteins from porcine milk by affinity chromatography

Results

1. There is one non-immunoglobulin protein in porcine milk that specifically bind to the LPS of *E.coli* F4. This protein has molecular weight of about 14 KDa and isoelectric point >11 (proteins number 1 on Fig. 2). Characterization of this protein is underway
2. Two other proteins (protein number 2 and 3 on Fig. 2) have strong attachment to the LPS than sepharose beads. Protein number 3 is lactoglobulin and characterization of protein numbe 2 is being performed.
3. Several other proteins bind to the sepharose beads and possibly to the LPS of *E.coli* F4.

Introduction

Recognition and binding to pathogens are crucial steps of the host defense against infectious diseases. These protective effects have been recognized in colostrum and milk and attributed predominantly to colostral and lactogenic immunoglobulins. Since there are many other non-immunoglobulin proteins in milk, objective of this study was to investigate non-immunoglobulin proteins in porcine milk that bind to LPS of *Escherichia coli* F4, an important enteric pathogen in piglets

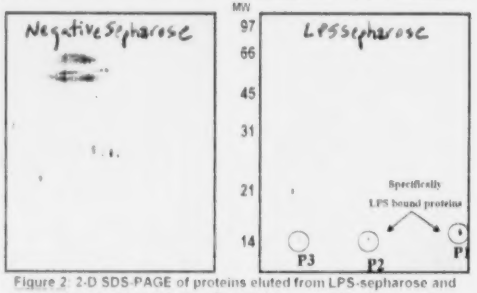

Figure 2: 2-D SDS-PAGE of proteins eluted from LPS-sepharose and sepharose (negative) columns. Protein 1 has specific affinity for LPS, whereas proteins 2 and 3 have affinity for both LPS and Sepharose.

Conclusion

This study demonstrated that, in addition to immunoglobulins, porcine milk has a repertoire of non-immunoglobulin LPS-binding proteins that may involve in innate defense against enteric diseases in suckling piglets.

Materials and Methods

Affinity chromatography: Lipopolysaccharide (LPS) isolated from *E.coli* F4 was coupled to CNBr Activated Sepharose 4B (Amersham Pharmacia Bio-Sciences) and subjected to 21 day fat-free porcine milk. Proteins bound to the LPS were eluted by 4 M Urea (Fig. 1). Affinity matrix alone was used as negative control to identify nonspecifically bound milk proteins to the matrix.

Protein detection: Eluted protein fractions were concentrated and detected by 2-dimensional electrophoresis (Fig. 2). Western blot was used to distinguish eluted immunoglobulins from the other LPS-binding proteins in porcine milk (Fig. 3).

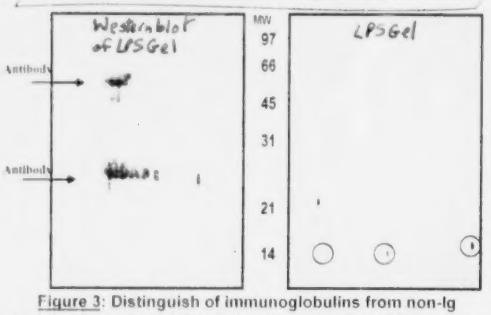

Figure 3: Distinguish of immunoglobulins from non-Ig proteins by western blot of eluted proteins from LPS column

Fundings

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Escherichia coli fimbrial-binding proteins in porcine milk

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Introduction

Neonatal diarrhea caused by *E.coli* F4 is one of the most important diseases in piglets. To cause diarrhea, *E.coli* must attach to the intestinal mucosa using fimbria.

Recognition and binding to pathogens are crucial steps of the host defense against infections. These protective effects have been attributed predominantly to immunoglobulins in porcine colostrum and milk.

Objective

The purpose of this study was to determine if non-immunoglobulin proteins in porcine milk bind to fimbria of *E. coli* F4.

Hypothesis

Non-immunoglobulin substances in porcine milk bind to fimbria of *E. coli* and prevent its attachment to the intestinal mucosa.

Material and Method

Affinity chromatography: Fimbriae isolated from *E.coli* F4 coupled to CNBr Activated Sepharose 4B were used for isolation of fimbrial-binding proteins from fat-free porcine milk (Fig. 1). Affinity matrix alone was used as the negative control to identify milk proteins that bind to the matrix.

Protein detection: Protein fractions eluted by 1M NaCl and 0.1 M Glycin pH 2.5 from both positive and negative columns were concentrated and characterized by Liquid Chromatography Mass Spectrometry (LCMS) (Table 1).

Results

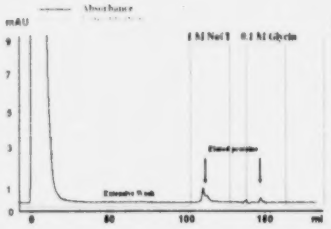

Figure 1. Isolation of fimbrial-binding proteins from porcine milk by affinity chromatography

Features / Protein	Eluted by	Physiological Function	Molecular Weight (Da)
Lactadherin	NaCl and Glycin	Binds proteins	45,725
Butrophilin	NaCl and Glycin	Membrane-associated receptor	59,276
Xanthin	NaCl	Cofactor binds Fe-S cluster	146,800
Ezrin (Villin2)	NaCl	Connects cytoskeleton to plasma membrane	68,629
Moesin	NaCl	Connects cytoskeleton to plasma membrane	67,529
Calmodulin	NaCl	Binds calcium	16,706
Alpha s2-Casein	NaCl	Transfers calcium	27,570
Beta-Casein	NaCl and Glycin	Casein Micelles	25,949
Fatty acid binding protein	NaCl and Glycin	Binds long chain and retionic acid	14,545

Table 1. Major porcine lactogenic non-imunoglobulin proteins that bind to the *E. coli* F4 fimbriae

Conclusions

- **More than 15 non-immunoglobulin proteins in porcine milk bind to the fimbriae of *E. coli* F4, but not to the sepharose matrix.**
- **Based on the elution procedures lactadherin, butrophilin, beta-Casein and fatty acid binding proteins bind more avidly to the *E.coli* F4 fimbriae than the other eluted proteins.**
- **These proteins may be involved in innate defense against enteric colibacillosis, by interfering with bacterial attachment to the intestinal mucosa.**
- **Studies to determine protective functions of these proteins are in progress.**

Funding

- **Saskatchewan Agriculture Development Fund**
- **Sask Pork and Agri-Food Inovation Fund**
- **Alberta Agriculture Research Institute**
- **Interprovincial Graduate Student Fellowship**

Non-immunoglobulin *Escherichia coli* F4 fimbrial and lipopolysaccharide-binding proteins in porcine milk

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Introduction

Diarrhea and septicemia/endotoxemia are the most important diseases in piglets. *E.coli* F4 is the most common cause of neonatal diarrhea. The first necessary step of neonatal intestinal colibacillosis is bacterial attachment to intestinal mucosa facilitated by fimbriae.

Septicemia or endotoxemia is initiated by presence of either Gram negative bacteria or their lipopolysaccharide (LPS), respectively, in the blood.

Recognition and binding to pathogens are crucial steps of the host defense against infections. In porcine colostrum and milk, these protective effects have been attributed predominantly to immunoglobulins and little is known about function of non-immunoglobulin substances in milk.

Hypothesis

Non-immunoglobulin substances in porcine milk bind to F4 fimbria and LPS of *E. coli* F4 and prevent attachment to the intestinal mucosa.

Objective

The purpose of this study was to determine if non-immunoglobulin proteins in porcine milk bind to F4 fimbria and LPS of *E. coli* F4.

Materials and Methods

Affinity chromatography: F4 Fimbria and LPS isolated from *E.coli* F4 coupled to CNBr Activated Sepharose 4B were used for isolation of fimbrial and LPS-binding proteins from fat-free porcine milk (Fig. 1). Affinity matrix alone was used as the negative control to identify milk proteins that bind to the matrix.

Protein detection: Protein fractions eluted by 1M NaCl pH 7.2 and 0.1 M glycin pH 2.6 from both positive and control columns were concentrated and characterized by Liquid Chromatography Mass Spectrometry (LC-MS) (Tables 1 and 2).

Results

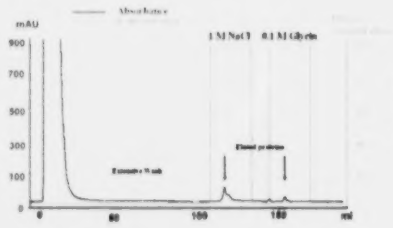

Figure 1. Isolation of fimbrial and LPS-binding proteins from porcine milk by affinity chromatography

	Fimbria Column		Control Column	
	NaCl elution	Low pH elution	NaCl elution	Low pH elution
Lactadherin	+*	+	-	-
β- Casein	+	+	-	-
Ezrin	+	-	-	-
Moesin	+	-	-	-
α- lactalbumin	+	-	-	-
Xanthin dehyd	+	-	-	-

* + : present - : absent

Table 2. Non-immunoglobulin F4 fimbria-binding proteins in porcine milk eluted from F4-sepharose but not from sepharose matrices by 1M NaCl and low pH buffers. Eluted proteins were identified by Liquid Chromatography Mass Spectrometry

	LPS Column		Control Column	
	NaCl elution	Low pH elution	NaCl elution	Low pH elution
Lactoferrin	+*	+	+	-
β- Casein	+	+	+	-
α-S2 Casein	+	-	-	-
κ- Casein	+	-	-	-
α-S1 Casein	+	-	-	-
Whey acidic prot	+	-	-	-
Lipoprot Lipase	+	-	-	-

* + : present - : absent

Table 1. Non-immunoglobulin LPS-binding proteins in porcine milk eluted from LPS-sepharose but not from sepharose matrices by 1M NaCl and low pH buffers. Eluted proteins were identified by Liquid Chromatography Mass Spectrometry

Conclusions

- There are non-immunoglobulin proteins in porcine milk that bind to F4 fimbria and LPS of *E. coli* F4.
- Lactoferrin and β-casein have higher affinity for LPS than other eluted proteins because these two proteins were not completely eluted by NaCl. Likewise lactadherin and β-casein have higher affinity for F4 fimbria than other proteins.
- The binding proteins may be involved in innate defense against enteric colibacillosis, by interfering with bacterial attachment to the intestinal mucosa.
- Studies to determine protective functions of these proteins are in progress.

Funding

- Saskatchewan Agriculture Development Fund
- Sask Pork and Agri-Food Inovation Fund
- Alberta Agriculture Research Institute
- Interprovincial Graduate Student Fellowship

Abstract

Neonatal diarrhea caused by *E. coli* F4 and septicemia/endotoxemia are the most important diseases in piglets. Fimbriae and endotoxin (lipopolysaccharide - LPS) are two virulence factors of *E.coli* facilitating attachment of bacteria to intestinal mucosa and induction of septicemia, respectively. Immunoglobulins present in colostrum and milk protect neonates against infections. However, function of the non-immunoglobulin components of the milk is not well characterized. We hypothesized that non-immunoglobulin substances in porcine milk protect suckling piglets from enteric pathogens. Using affinity chromatography we investigated if non-immunoglobulin substances bind to F4 fibmria and LPS of *E. coli* F4. Results showed that a number of proteins in porcine milk such as lactadherin, xanthin, ezrin and beta casein bound to the *E. coli* F4 fimbria and proteins such as lactoferrin, alpha casein, and beta casein bound to the *E. coli* F4 LPS. Our results indicate that in addition to immunoglobulins, there are many substances in porcine milk that bind to F4 fimbria and LPS, which might be involved in innate defense against bacterial colonization of intestinal mucosa.